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3- nitropropionic acid-induced ischemia tolerance in the rat brain is mediated by reduced metabolic activity and cerebral blood flow

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Abstract

Tissue tolerance to ischemia can be achieved by noxious stimuli that are below a threshold to cause irreversible damage (“preconditioning”). Understanding the mechanisms underlying preconditioning may lead to the identification of novel therapeutic targets for diseases such as stroke. We here used the oxidative chain inhibitor 3-nitropropionic acid (NPA) to induce ischemia tolerance in a rat middle cerebral artery occlusion (MCAO) stroke model. Cerebral blood flow (CBF) and structural integrity were characterized by longitudinal Magnetic Resonance Imaging (MRI) in combination with behavioral, histological, and biochemical assessment of NPA preconditioned animals and controls. Using this approach we show that the ischemia tolerant state is characterized by a lower energy charge potential (ECP) and lower CBF, indicating a reduced baseline metabolic demand, and therefore a cellular mechanism of neural protection. Blood vessel density and structural integrity were not altered by NPA treatment. When subjected to MCAO, preconditioned animals had a characteristic MRI signature consisting of enhanced CBF maintenance within the ischemic territory and intra-ischemic reversal of the initial cytotoxic edema, resulting in reduced infarct volumes. Thus, our data show that tissue protection through preconditioning occurs early during ischemia and indicate that a reduced cellular metabolism is associated with tissue tolerance to ischemia.

Key words: Acute Stroke, Animal Models, ASL, Brain Imaging, MRI

Introduction

The ability of an organism to develop tolerance against stressful conditions can help to survive temporary restrictions in energy supply, low oxygenation, or other environmental challenges. Among other organs, the brain can acquire tolerance to experimental injury such as trauma or ischemia (1). In human stroke, the immediate onset and time-dependent course of damage have driven the search for rapid neuroprotective measures. The interest in ischemia tolerance was fueled by clinical observations that a brief episode of ischemia such as a transient ischemic attack of the brain or preinfarction angina of the heart, might protect against subsequent stroke or heart attack, similar to experimental preconditioning (2-4). To study mechanisms involved in ischemia tolerance, several experimental preconditioning strategies have been described *in vivo* and *in vitro*, such as hypoxia, brief episodes of ischemia or oxygen-glucose deprivation (5-8). Some of the mechanisms identified include attenuation of excitatory damage through reduced glutamate release and downregulation of NMDA receptors, changes in gene expression patterns favoring neuroprotection, as well as neurorestorative responses such as the induction of neural progenitor cells. Although vascular adaptations in response to preconditioning could help to facilitate substrate delivery during subsequent ischemia, the potential role of blood vessel remodeling in ischemia tolerance remains open (5, 9).

Our goal was to characterize mechanisms of preconditioning and to predict histological and behavioral outcomes after stroke by defining an MRI signature of the ischemia tolerant brain. We utilized a pharmacological preconditioning paradigm with the oxidative chain inhibitor NPA, which temporarily induces ischemia tolerance through inhibition of succinate dehydrogenase (SDH) and a burst of reactive oxygen species (ROS) (10-14). Using longitudinal, quantitative arterial spin labeling (ASL)-CBF measurements as well as structural MRI we here show that ischemic damage and recovery evolve differently in ischemia tolerant brains. CBF was significantly reduced in NPA-preconditioned rats at the time of ischemia

tolerance, which was associated with decreased metabolic activity, as determined from the quantification of high energy phosphates in tissue obtained from NPA-treated and control rats. Thus, we here identify a cellular mechanism mediating preconditioning in the context of ischemic stroke.

Materials and Methods

Preconditioning, stroke induction and experimental groups

All experiments were performed in accordance with the National Institutes of Health animal protection guidelines and approved by the UCSD Institutional Animal Care and Use Committee (IACUC). Adult male Wistar rats 280 – 310 mg body weight were used for all experiments.

For preconditioning, NPA (20mg/kg bw) or the same volume of saline (controls) was injected once intra-peritoneally. Three days after sham or NPA treatment, animals were subjected to 60 min of MCAO. A modification of the Koizumi intraluminal filament - method was applied (15). During surgery and MRI, animals were anesthetized using facemask inhalation of 1.5-2.5 % isoflurane in a 2:1 N₂O:O₂ atmosphere. Temperature was maintained at 37°C by a feedback-controlled heating pad.

The first MRI (D0) was acquired during MCAO without discontinuation of anesthesia. For MRI, the animal was placed in a custom-built cradle. Body temperature and respiratory rate were continuously monitored during MRI sessions. After 60 min of MCAO, the occluding filament was removed and the animals allowed to recover. Animals were re-anesthetized for repetitive MRI at the following time points after MCAO: day 0 (D0), D1, D4, and D14 after MCAO. Vasodilatory capacity was assessed on D4 and D14 by comparing MRI-CBF measurements acquired under isoflurane anesthesia in room air (“air”) with those acquired in a 5% CO₂ atmosphere (“CO₂”), after a 2 minute adjustment between switching gases as previously described (16). Animals were sacrificed on D14 after the last MRI acquisition and brains subjected to further histological analysis. The CBF maps obtained from the control group have been published elsewhere (17). Functional assessments were obtained before each MRI session and in addition on D7 after MCAO. Of the initially included 21 animals, eight controls and five NPA animals remained in both groups; exclusions were due to massive stroke and intolerable weight loss/behavioral abnormalities/seizures: controls n = 3, NPA n =

0; due to surgical complications (subarachnoid hemorrhage upon advancement of the thread): controls $n = 2$, NPA $n = 1$; and due to behavioral abnormalities such as reduced spontaneous activity or failure to perform baseline functional assessment within predefined range (see below): controls $n = 0$, NPA $n = 2$. Another group of animals received either i.p. NPA ($n = 7$) or saline ($n = 12$) treatment and MRI assessment three days later. Following MRI, animals were sacrificed and brains extracted for further histological analysis. A separate group of animals was treated with either NPA ($n = 10$) or saline ($n = 10$) and brains extracted for high-performance liquid chromatography (HPLC) analysis of brain energy rich phosphates three days later.

MRI acquisition and data processing

MRI experiments were carried out on a 3 T GE Signa Excite whole-body system with a body transmit coil and a custom-built passively decoupled single-loop receive-only head coil. CBF imaging was achieved with multislice flow-sensitive alternating inversion recovery (FAIR) pulsed arterial spin labeling (pASL) with the QUIPSS II modification (18). In addition, diffusion-weighted imaging (DWI) with generation of apparent diffusion coefficient maps (ADC) and fractional anisotropy (FA) maps, as well as T1- and T2 - weighted imaging were carried out. For details of the MRI sequences and analysis strategies please see also (17, 19).

Images from all animals at all time-points were first co-registered and then averaged using codes written in Matlab (Mathworks, Natick, MA, USA) software. Vasoreactivity (VR) maps were generated by subtracting the CBF map acquired in air from the one acquired in 5% CO₂. Voxels with significantly reduced ADC values were automatically selected as all voxels with a value of $< \text{mean} + 2 \text{ standard deviations (SD)}$ of a contralateral hemisphere region of interest (ROI). Similarly, ROIs for increased T2-values ($> \text{mean} + 2\text{SD}$ contralateral on a T2 map) or hyperperfusion ($> \text{mean} + 2\text{SD}$ contralateral on a CBF map) were selected. A voxel-

wise independent sample t-test was used to compare CBF maps of NPA or with those of control animals with a threshold of $p < 0.05$ using Matlab codes.

Behavioral testing

Adhesive tape removal test: to assess sensorimotor function and neglect after MCAO, two strips of tape (18 x 12 mm) were applied in random order to the saphenous part of the forepaws. The time animals took to contact (sensory function/neglect) and remove (sensorimotor function) the tape on both sides (left and right) was recorded. Results from three trials at least 5 min apart were averaged at each session (20, 21).

The 18-point observational score for assessment of sensorimotor deficits after MCAO was also applied; results from this score for the control group have been previously published (17, 22). In this test, a maximum of three points is scored for each of the following observations: (1) spontaneous activity, (2) symmetry in limb movement, (3) forepaw outstretching, (4) climbing, (5) body proprioception, and (6) response to vibrissae touch, so that 18 points indicate no neurological deficit.

Cerebral tissue processing and HPLC analyses of metabolites

An in vivo craniectomy was performed on all animals during anesthesia with the aid of an operating microscope. After carefully removing the rat's skull, the brain was freeze-clamped by aluminum tongues, pre-cooled in liquid nitrogen, and immediately immersed in liquid nitrogen, to accelerate freezing of the tissue and increase the recovery of metabolites (23). After the wet weight (w/w) determination, the brain was deproteinized according to the organic solvent deproteinizing procedure, described in details elsewhere, which ensures no loss of labile compounds and guarantees an efficient deproteinization of the tissue for metabolite analysis (24). Deproteinized samples were then filtered through a 0.45- μ m HV Millipore filter (Millipore Corp., Billerica, MA) and loaded (20 μ L) onto a Hypersil C-18,

250 x 4.6 mm, 5- μ m particle size column, provided with its own guard column (Thermo Fisher Scientific, Milan, Italy). The column was connected to a HPLC apparatus consisting of a SpectraSystem P4000 pump system (Thermo Fisher Scientific) and a highly-sensitive UV6000LP diode array detector (Thermo Fisher Scientific) equipped with a 5-cm light path flow cell and setup between 200 and 300 nm wavelengths. Data acquisition and analysis were performed using a personal computer and the ChromQuest software package provided by the HPLC manufacturer. NAA and metabolites related to tissue energy state and mitochondrial function (ATP, ADP, AMP, NAD⁺) were separated, in a single chromatographic run, according to a gradient modification of existing ion-pairing HPLC method (24, 25). Assignment and calculation of the compounds of interest in chromatographic runs of tissue extracts were carried out at either 206 (NAA) or 260 nm wavelength by comparing retention times, absorption spectra, and areas of peaks with those of peaks of chromatographic runs of freshly prepared ultrapure standard mixtures with known concentrations. The ECP in brain extracts was calculated according to the equation: $ECP = ATP + 1/2ADP / ATP + ADP + AMP$.

Immunofluorescence and - histochemistry

After perfusion fixation, brains were removed, immersed in ice-cold 4% formalin and transferred to 30% sucrose solution for at least 3 days. Coronal 40- μ m-thick sections were cut on a freezing microtome (Leica, Nussloch, Germany). Every twelfth slice from each brain was stained for KCC2 and RECA-1 fluorescence intensity measurement. All sections were stained and imaged in parallel. Primary antibodies used were mouse α -RECA-1 1:400 (AbD SEROTECH), rabbit α -KCC2 1:500 (Millipore), α -rabbit CC3 1:400 (Bioconcept), α -rabbit GFAP 1:1000 (Sigma), α -mouse MAP2 1:500 (Abcam), α -mouse COX-IV 1:250 (Abcam). Secondary antibodies were goat α -mouse-Cy3 1:300 and goat α -rabbit-Alexa488 1:300

(Jackson ImmunoResearch). Nuclei were counterstained with 4-6-diamidino-2-phenylindole (DAPI, Sigma). Images were processed in Adobe Photoshop (CS5 for Mac) or ImageJ (NIH).

For DAB immunohistochemistry, endogenous peroxidase was quenched using 0.6% hydrogen peroxidase for 30 minutes at RT, following the regular staining protocol using mouse α -NeuN 1:200 (Millipore), α -ED1 1:1000 (Serotec) as a primary antibody and mouse α -Biotin (1:250; Jacksonimmuno) as a secondary antibody. DAB staining was done using VECTASTAIN Elite ABC Kit (Vector laboratories) and DAB Peroxidase Substrate Kit, 3, 3'-diaminobenzidine (Vector laboratories). Sections were mounted using DPX Mountant (Sigma).

Image analyses

For fluorescence intensity quantification, images were taken using a 20x objective with an inverted Leica Wide field microscope (Leica DMI 6000, Leica). ROIs (710x530 μ m) were chosen from cortical and subcortical regions bilaterally, using the same settings for all conditions. The measurements from contralateral and ipsilateral ROIs were combined per section and animal. RECA-1 and KCC2 fluorescence intensity was measured and calculated using the ImageJ (NIH) software package as mean signal intensity/ mm^2 \pm SD.

For the quantification of NeuN- DAB staining after stroke, areas showing loss of NeuN expression were derived by subtracting the manually outlined areas of intact NeuN+ staining on the ipsilateral side from the area of the contralateral hemisphere, thereby correcting for edema. The sum of all areas with loss of NeuN staining was multiplied by the distance between slices, deriving lesion volume in mm^3 .

COX and KCC2 signal intensity were measured with a frame size of 1024×1024 pixels on a scanning confocal microscope (FluoViewTM FV1000, Olympus) at a constant Z-step of 0.5 μ m using a 20x objective for COX and 40x oil objective for KCC2. Quantification was done using ImageJ (NIH). All images were taken with the same laser power and gain settings.

Pictures were taken contralateral and ipsilateral from a cortical, striatal and dentate gyrus (DG) region, per section and animal. All pictures were analyzed in Imaris 7.6.3 (Bitplane) using the Imaris MeasurementPro package (Bitplane). The MAP2 signal was 3d reconstructed while using the same threshold settings for all images per region. The volume was calculated and the KCC2 intensity was extracted from the MAP2 3d reconstruction.

Statistical analysis

All statistical analyses were carried out in SPSS v12.0 for Windows, except for the voxel-wise t-test of CBF data (Figure 4A), which was performed in Matlab.

All values are given as mean +/- standard deviation (SD). For group comparisons, the two-sided independent sample t-test was used. In not normally distributed data, the nonparametric Mann-Whitney U test was used for group comparisons. Repeated measures general linear model analyses were conducted to assess significant differences between functional performances over time in control or NPA animals. A p-value < 0.05 was considered significant.

Results

NPA preconditioning results in tissue protection and improved functional outcome after MCAO through maintained intra-ischemic CBF and ADC recovery

Experimental ischemia was induced by MCAO in adult male Wistar rats preconditioned with NPA or pretreated with saline. Fifteen min after occlusion of the MCA, ischemic lesions were readily detected on DWI, affecting cortical and subcortical areas within the occluded left MCA-territory in both groups (Figure 1 A). Notably, in a second DWI acquisition 30 min later with the occluding device still in place, lesions had remained stable in controls, but had diminished in NPA-treated rats, where only one animal showed a significant reduction in the apparent diffusion coefficient (ADC) after 45 min of ischemia (Figure 1 A, B). Quantitative CBF imaging using ASL showed a drop in CBF on the ischemic side during MCAO in both groups and a recovery of CBF after retraction of the occluding filament from D0 until D14 (Figure 2 A). The perfusion restriction was, however, less severe in NPA animals (Figure 2 B). Between D1 and D14, all animals of the control group showed a post-ischemic hyperperfusion, which was significantly attenuated and only detected on D1 in the NPA group (Figure 2 A, C). This pattern of less extensive post-ischemic hyperperfusion with an earlier peak after reperfusion is an indicator of less severe ischemic damage (17). Indeed, infarct size as determined from T2 weighted MRI on D14 was dramatically smaller in preconditioned animals (Figure 2D). Diffusion tensor imaging (DTI) revealed that callosal and descending corticospinal fiber tracts were initially damaged, reflected by a hyperacute increase and subacute decrease in fractional anisotropy (FA), but, in contrast to controls, regained structural integrity in the NPA group (Figure 2 E).

The vasoreactivity remained intact in NPA preconditioned animals on D4 and D14 after MCAO. In contrast, the normal vasodilatory response to a 5% CO₂ stimulus was lost on the ischemic side in controls on D4 (Figure 2 F, G). In most control animals, vasoreactivity subsequently recovered or even showed an overshooting CBF-increase on D14.

MRI findings correlated with improved functional performance. In a composite neurological score, combining measures of sensory and motor function typically affected after MCAO, deficits were still detectable on D14 in controls, showing a significantly better performance of the NPA group at all observation time points (Figure 3A).

Deficits in sensorimotor function and neglect after MCAO were also assessed in the sticky tape test. The time to first contact and removal of the tape was measured. Before MCAO, animals were trained to remove the tape within 10 seconds. On D1 after left-sided MCAO, deficits to perceive and remove the tape on the right side were noted in all animals, causing an increase in the time to contact and remove the tape on the right forepaw (Figure 3 B). Both groups showed a fast recovery towards D7. On average, NPA animals performed faster, which did, however, not reach significance in the repeated measures ANOVA.

Tissue protection by NPA preconditioning

Next, we analyzed infarct size after MCAO in NPA-treated vs. control rats. Loss of NeuN-expression was significantly smaller in NPA than in control animals ($13.3 \pm 2.5 \text{ mm}^3$ vs. $48.6 \pm 11 \text{ mm}^3$; $p < 0.01$ Figure 4 A, B, C). Strikingly, loss of NeuN expression occurred in most NPA animals without signs of tissue necrosis, while controls had undergone extensive cystic degeneration of brain tissue on the ischemic side (Figure 4 A, C). In controls, infarct border zones were characterized by loss of NeuN staining, detection of cleaved caspase-3 (CC3) positive cells indicating apoptosis, an increase in GFAP signal due to gliosis, and an increase in RECA-1 signal corresponding to peri-infarct angiogenesis. Furthermore, macrophage infiltration around the infarct border zone was visible on ED1 immunohistochemistry (Figure 4 A, D).

Neuronal death was observed in all NPA animals after MCAO in scattered cortical and subcortical regions, along with GFAP and ED1 positivity, indicating gliosis and macrophage activation (Figure 4 A, C, D). Of note, these areas were not detected on T2w MRI, which is

the MRI parameter most commonly applied to quantify chronic ischemic damage (26). This indicates that NPA induced neuroprotection after stroke is not complete and that regional loss of neurons may occur.

In search for mechanisms of NPA-induced ischemia tolerance, we further analyzed the expression of the K^+/Cl^- co-transporter KCC2 in brains of animals subjected to MCAO with and without NPA preconditioning. KCC2 is upregulated in response to excitotoxic injury, maintaining ion gradients and supporting neuroprotection (27, 28) and downregulated in response to more severe ischemia (29, 30). By preventing excessive intracellular Cl^- accumulation and hyperexcitability after ischemia, KCC2 upregulation might contribute to a protective response elicited by preconditioning. We did not find a specific alteration of the KCC2 expression in preconditioned brains after ischemia; but a decrease in KCC2 signal in the infarct area of both, controls and NPA-preconditioned animals after MCAO (Figure 4 A), indicating that post-ischemic downregulation of KCC2 occurs despite preconditioning in the areas of neuronal damage, most likely reducing GABAergic inhibitory functions.

Structural and metabolic adjustments after NPA preconditioning

After the robust neuroprotective effect of NPA preconditioning was confirmed in the rat MCAO model, we next sought to characterize changes in the vascular and metabolic properties of animals in the ischemia tolerant state. In analogy to the MCAO experiments, two groups of animals were either preconditioned with i.p. NPA or saline. Three days later animals were not subjected to MCAO, but analyzed using MRI and histological analyses of brain tissue. While NPA - preconditioning had no impact on the gross structural integrity, we found a global, approximately 30% reduction in baseline CBF in the NPA group (138.75 ± 19.2 vs. 108.6 ± 16.3 mg/100ml/min; Figure 5A). Despite this decrease in CBF, vasoreactivity was preserved (Figure 5B). Another cohort of animals with NPA-preconditioning and controls were subjected to HPLC analysis for quantification of metabolites related to

mitochondrial function and cell energy state, including adenosine nucleotides (ATP, ADP and AMP), N-Acetylaspartate (NAA) and (nicotinamide adenine dinucleotide) (NAD^+). In comparison to the saline group, three days after NPA preconditioning, tolerant animals had a non-significant decrease in ATP (2139.64 ± 220.24 vs. 2326.70 ± 180.00 nmol/g w.w.), equal ADP values (259 ± 90.1 vs. 207.4 ± 34.5 nmol/g w.w.), a significant increase in AMP (70.51 ± 8.47 vs. 47.19 ± 8.28 nmol/g w.w.; $p < 0.001$) and an unaltered sum of nucleotides (ATP + ADP + AMP). The change in the concentrations of adenylates caused a significant decrease in the energy charge potential (ECP: 0.919 ± 0.016 vs. 0.942 ± 0.004 ; $p < 0.01$), indicating a mitochondrial-dependent imbalance in energy production and consumption (Figure 5 C). NAA, a marker of neuronal integrity, was not significantly different between groups (NPA 8075.2 ± 971.7 vs. con 8783.4 ± 513.6), nor was the concentration NAD^+ (NPA 527.5 ± 68 vs. con 550.2 ± 39.7 nmol/g w.w.).

These results suggest that metabolic adjustments might underlie the observed lower CBF in ischemia tolerant NPA-treated animals. This was supported by a reduced expression of the respiratory chain enzyme cytochrome c-oxidase (COX) in NPA preconditioned brains (Figure 6 A, B). To assess possible adaptations at the vascular level, brains of NPA-preconditioned animals and controls underwent immunofluorescence analysis of the endothelial marker RECA-1 three days after treatment. We did not find a significant difference in RECA-1 expression between groups in any of the regions analyzed (cortex, striatum, dentate gyrus; Figure 6 A, B). We again analyzed brain KCC2 expression three days after NPA preconditioning without MCAO. We found no significant difference in KCC2 expression between NPA animals or controls in any of the three regions analyzed (Figure 6 A, B). This argues against a role of KCC2 as part of the NPA preconditioning signal. No signs of neuronal damage were detected in both groups with NeuN, MAP2- or caspase-3 stain for apoptotic cells (data not shown).

Discussion

The mechanisms by which a preconditioned brain maintains tolerance to ischemia remain obscure, especially the contribution of vascular versus metabolic adaptations. We approached this problem by analyzing CBF and metabolic phosphates in NPA preconditioned rats at the time of ischemia tolerance. Furthermore, infarct growth and functional outcomes were assessed after MCAO in preconditioned and sham-preconditioned rats.

MRI during and after MCAO in the NPA-mediated model of pharmacological preconditioning revealed that NPA-mediated protection of the brain started immediately during the ischemic challenge through maintenance of intra-ischemic blood flow and recovery of the initial MCAO-induced cytotoxic edema. As a consequence, structural tissue integrity was preserved, which was reflected by only slight and temporary disruption of fiber tracts in DTI, normal vasoreactivity, and less vasogenic edema with significantly smaller final T2-lesions. When CBF was measured in the ischemia tolerant animals (72 hours after NPA preconditioning), we found a significant global reduction in CBF with preserved vasoreactivity. Strikingly similar to our results, in a rat model of ischemia tolerance induced by cortical spreading depression (CSD), autoradiography demonstrated a CBF reduction restricted to those regions affected by CSD to a similar extent and at a similar time (72 hours after CSD) before ischemia (31). Laser Doppler flowmetry was suggestive of preserved intranscortical CBF in tolerant animals; however, the limitations of this technique including a lower spatial resolution and relative CBF measurements led the authors to hypothesize that based on the lower pre-ischemic CBF, absolute values might not have been higher in CSD preconditioned animals. These findings support a common mechanism of ischemia tolerance where metabolic adaptations are first installed which later facilitate preserved vascular responses to ischemia. Only few studies have investigated changes in CBF due to preconditioning before and at the time of subsequent ischemia. After preconditioning, some groups have found an increase in relative CBF, as determined from laser Doppler flowmetry,

during or in the hours following subsequent ischemia (32-34), while others observed neuroprotection without CBF effects during MCAO (35-37). Dawson et al. used autoradiography to show that 15 min after the onset of MCAO, CBF was unchanged in animals subjected to lipopolysaccharide preconditioning; however 4 and 24 hours later, microvascular perfusion as assessed by an intravascular fluorescent tracer technique was preserved at higher levels in preconditioned animals compared to controls (38). Gustavsson et al. observed an attenuation of the CBF decrease during a hypoxic/ischemic injury 24h after hypoxic preconditioning, along with an increase in microvascular density (9), albeit in a neonatal model where angiogenesis is likely to remain more plastic compared to the adult brain. In our model, no increase in vascularization was observed in ischemia tolerant brains, however, the lower ECP and reduced COX-expression pattern suggested a significant decrease in mitochondrial phosphorylation capacity at the rate necessary to meet the cellular energy demand subsequent to NPA administration. Additionally, the decrease in the rate of ATP synthesis provoked a change in the proportion of adenosine nucleotides suggesting a state of energy imbalance that has been shown to be associated with induction of cellular programs for neuroprotection (39). Our finding of decreased ECP together with the reduction of CBF throughout the brain of NPA preconditioned animals is a strong argument supporting a role of energy metabolism imbalance for preconditioning, as suggested by the tendency to decrease the rate of ATP synthesis with a concomitant increase in its dephosphorylated products (AMP). Since metabolites in our experiments were measured in whole brain homogenates, it is possible that the apparently modest differences found in these parameters might have been of larger amplitude in a small metabolically active compartment such as neuron terminals.

Albeit to a much lesser extent, such response might be similar to the prolonged energy conserving phase of torpor in hibernating animals (40). The analogy between ischemia tolerance in hibernators and tolerance induced by preconditioning has long been suggested by

similar neuroprotective mechanisms, such as the induction of HIF-dependent pathways, adenosine receptor activation and the reduction of glutamate release (1, 41). However, the role of metabolic downregulation in preconditioning-induced tolerance has remained open (5, 42-44). Based on our results, it is not possible to discriminate if the observed metabolic adjustments occurred directly due to the inhibition of SDH by NPA, or later. Interestingly, while the inhibition of SDH after NPA application is immediate (30 min) and longer lasting (3 days), the direct effect of NPA on ATP synthesis is much shorter lasting (approx. 2 hours) (11).

Histological analysis proved the NPA-induced tissue protection suggested from MRI, however, selective neuronal death occurred in parts of the ischemic territory that were not detected on T2w MRI. This finding points out the importance of multimodal (imaging/ functional testing/ histology) readouts in the assessment of ischemic damage. Although our findings may be specific for preconditioning with NPA, evidence from other preconditioning paradigms speak for common principal mechanisms, such as induction of Bcl-2, ATP-dependent potassium channels and TNF α (7, 45, 46). Future experiments will be needed to test additional models of preconditioning using longitudinal, quantitative CBF measurements. Current outcome prediction algorithms for stroke are based on preexisting, non-modifiable patient characteristics (47). Based on the findings presented here, MRI-based approaches will be able to refine these parameters and have the potential to guide future therapeutic decisions by analyzing pre-ischemic or intra-ischemic CBF, post-ischemic hyperperfusion and early DWI lesion dynamics.

Disclosure/Conflict of Interest

The authors of this manuscript have no conflict of interest to disclose.

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Titles and legends to figures

Figure 1: Intra-ischemic ADC recovery in NPA-preconditioned animals

A) Masks of the ischemic lesion from individual animals of the control (Con) or NPA-treated group derived from ADC maps acquired 15 (upper panel) or 45 (lower panel) minutes after the onset of MCAO. Masks are overlaid onto anatomical (T2w) images. Regions of overlap between individual animals are color-coded. While the area of ADC reduction is similar at 15 and 45 min ischemia duration in controls, there is a dramatic intra-ischemic recovery in the NPA group. B) Number of voxels with a significantly reduced ADC (mean \pm SD) at 15 and 45 min after the onset of ischemia (** $p < 0.01$).

Figure 2: NPA preconditioning results in less severe ischemic perfusion restriction and attenuated tissue damage following stroke.

A) Averaged CBF maps from controls (Con) or NPA-treated animals during ischemia (D0) and on days 1, 4 and 14 (D1, D4, D14) demonstrate an attenuated drop in CBF on D0 in the NPA group (red arrow) compared to controls (white arrow). Post-ischemic hyperperfusion in the NPA group is only observed in few voxels on D1 (red arrow) compared to controls (white arrow).

B) CBF < 30 : voxels with a CBF value below 30 ml/100g/min on D0, resembling severely reduced perfusion. There are significantly less voxels with intra-ischemic CBF < 30 in the NPA- compared to the control group (* $p < 0.05$).

c) Number of voxels with post-ischemic hyperperfusion (CBF $>$ mean + 2SD contralateral) in controls (dark grey columns) and NPA group (white column) at D1, D4 and D14 after

reperfusion. There are significantly more hyperperfused voxels in controls than in the NPA-group; the latter with hyperperfusion only on D1 (** $p < 0.011$; D4 and D14 not shown for NPA).

D) The number of voxels with an increased T2 value ($> \text{mean} + 2\text{SD}$ of contralateral), indicating infarct, is significantly higher in controls (** $p < 0.001$) than in NPA animals. To the right, averaged T2 maps from D14 show high signal on the ischemic side in controls, whereas there is barely any signal abnormality detectable in the NPA group.

E) Averaged fractional anisotropy (FA) maps from DTI overlaid onto averaged ADC maps on D0, D1, D4 and D14 in the control and NPA group. After a brief episode of increased FA during ischemia (red asterisks), FA is decreased within the area of ischemia (white arrows), indicating derangement of fiber tracts. However, while FA remains low in controls, remaining fiber tracts show a recovery of FA values in NPA animals (red arrow). Also note the development of vasogenic edema, causing an increase in ADC values on the ischemic side in controls, but not in the NPA group.

F) Averaged vasoreactivity maps, derived from subtraction of the CBF map acquired during inhalation of air from the CBF map acquired with 5% CO₂ as a vasodilatory stimulus on D4 and D14 in controls and NPA animals. White arrows point to a lack of CBF increase due to 5% CO₂ within the ischemic area in controls on D4, indicating impaired vasoreactivity, which has recovered on D14. Note the overshooting vasoreactivity in peri-lesional, cortical areas in controls on D14, which is present in some but not all animals of the control group. In NPA animals, vasoreactivity within the ischemic area is maintained. G) Vasoreactivity expressed as % increase in CBF with the 5% CO₂ stimulus on D4 and D14 within the voxels with a CBF $< 30\text{mg}/100\text{ml}/\text{min}$ at D0 (severely ischemic voxels). In contrast to NPA preconditioned animals, vasoreactivity was significantly impaired on D4 in controls (** $p < 0.01$).

Figure 3: Sensorimotor deficits after MCAO are attenuated by preconditioning.

A) In a composite neurological score where 18 points indicate no deficits and lower scores an impairment of sensorimotor function, NPA (light gray line) animals are significantly less affected than controls (black line) (** $p < 0.01$).

B) Latency to contact (dashed lines) and remove (solid line) a sticky tape applied to the left or right (indicated in upper right corner of the diagram) forepaw of rats in the control (black) or NPA (light gray) group at different time points before (D0) and after (D1, D4, D7, D14) MCAO. Due to occlusion of the left MCA, the time to contact or remove the tape on the right side is longer in all animals after ischemia. However, on average, deficits are less severe in the NPA group (this effect did not reach significance in repeated measures ANOVA).

Figure 4: Ischemic lesions after MCAO in controls and NPA-preconditioned animals

A) Representative immunofluorescence images demonstrating expression of NeuN (green), CC3 (red), GFAP (red), RECA-1 (green), and KCC2 overlaid onto DAPI (red and blue) on the ipsilateral (stroke) and contralateral (contra) side in controls (upper and middle panel) as well as on the ipsilateral (stroke) side in NPA preconditioned animals (lower panel). Two weeks after MCAO, tissue necrosis has occurred in controls, while only selective neuronal damage can be detected in NPA animals (white arrow on the lower panel NeuN staining). B) Infarct size derived from NeuN-immunohistochemistry in Con and NPA animals. Note that this analysis includes areas of cystic tissue degeneration as well as selective neuronal loss. C) Left: overview (2x) of NeuN immunohistochemistry in a Con (upper panel) versus NPA (lower panel) animal. Right: Higher (10x) magnification of the NeuN immunohistochemistry in a control animal showing normal striatal NeuN expression on the contralateral (upper panel) and loss of NeuN expression on the ipsilateral (lower panel) side. D) ED1-

immunohistochemistry demonstrating macrophage infiltration at the infarct border in controls (upper panel) and at the zones of selective neuronal death in NPA animals (lower panel).

Figure 5: The ischemia-tolerant state is characterized by lower baseline CBF and energy imbalance in NPA-preconditioned animals.

A) Average CBF maps from controls (Con) and NPA-preconditioned (NPA) animals three days after saline or NPA treatment without the induction of experimental stroke. Lower panel: voxel-wise statistics of CBF values between groups indicating voxels with significantly lower CBF in NPA animals in pink, overlaid onto anatomical (T2w) images. There is a global CBF reduction three days after NPA treatment compared to controls.

B) Comparison of whole-brain CBF values between groups with and without CO₂ challenge. Although baseline CBF is lower in NPA animals, CBF increases to a similar extent after application of CO₂, which indicates that vasoreactivity is preserved after NPA treatment.

C) Levels of the brain metabolites ATP, AMP and ECP three days after NPA preconditioning or sham treatment. There is significantly more AMP and a lower ECP in brains of the NPA group.

Figure 6: NPA preconditioning decreases the expression of COX, but does not influence the density of blood vessels or the expression of the chloride co-transporter KCC2.

A) Panels are showing MAP2 (green), KCC2 (red), RECA-1 (red) and COX (red) expression in the cortex (upper panels), striatum (middle panels) and dentate gyrus (DG, lower panels) of an exemplary control and NPA animal. Nuclei were counterstained with DAPI (blue). MAP2: MAP2 expression (green), KCC2 in MAP2: KCC2 signal (red) extracted from the MAP2-

masked area, Merge: overlays of MAP2, KCC2 and DAPI. RECA-1: overlay of RECA-1 expression (red) and DAPI (blue). COX: overlay of COX expression (red) and DAPI (blue).

B) Quantitative analyses of the KCC2 (left), RECA-1 (center), and COX (right) immunofluorescence signal in cortex, striatum and DG in controls and NPA (mean \pm SD).